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Rapid, Potentially Automatable, Method Extract Biomarkers for HPLC/ESI/MS/MS to Detect and Identify BW Agents

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RAPID, POTENTIALLY AUTOMATABLE, METHOD EXTRACT BIOMARKERS FOR HPLC/ESI/MS/MS TO DETECT AND IDENTIFY BW AGENTS

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ABSTRACT

Our program proposes to concentrate on the rapid recovery of signature biomarkers based on automated high-pressure, high-temperature solvent extraction (ASE) and/or supercritical fluid extraction (SFE) to produce lipids, nucleic acids and proteins sequentially concentrated and purified in minutes with yields especially from microeukaryotes, Gram-positive bacteria and spores. Lipids are extracted in higher proportions greater than classical one-phase, room temperature solvent extraction without major changes in lipid composition. High performance liquid chromatography (HPLC) with or without derivatization, electrospray ionization (ESI) and highly specific detection by mass spectrometry (MS) particularly with $(MS)^n$ provides the detection, identification and because the signature lipid biomarkers are both phenotypic as well as genotypic biomarkers, insights into potential infectivity of BW agents. Feasibility has been demonstrated with detection, identification, and determination of infectious potential of *Cryptosporidium parvum* at the sensitivity of a single oocyst (which is unculturable in vitro) and accurate identification and prediction, pathogenicity, and drug-resistance of *Mycobacteria* spp.

INTRODUCTION

The threat of a terrorist attack based on biological weapons has been widely reported as a major threat to international security. The catastrophic potential of such an attack cannot be overstated. Of particular concern is the great potential for harm incurred by genetically engineered microbes. The "anthrax" episode in June 1997 in Washington DC, brought to light the inadequacy of the methodology currently employed to rapidly assay for the presence and nature of biological weapons.

Over the past 20 years, our laboratory has developed the signature lipid biomarker (SLB) method of analysis for the quantitative definition of the viable biomass, community composition, and nutritional status of microbiota isolated from a wide variety of environmental matrices including air, soil, wounds, and water [1]. In many cases, unique molecules specific to microorganisms that could be useful in detecting, identifying, and determining the infectious potential of life threatening microbial pathogens have been detected. Microbes or microbially derived imunomodulating agents can be concentrated

from air or water as aerosol or aqueous filter retentates, or recovered directly from environmental matrices like sediments, soils, vectors, and clinical specimens and detected/identified from analysis of lipid components. All independently viable agents or agents requiring cell hosts must have an intact polar lipid membrane for morbidity. With new extraction techniques additional biomarkers including nucleic acids and proteins could be added to the lipids thereby providing additional specificity to the detection systems.

The analysis of biomarkers involves four major processes: concentration of the microbes or their components from the environmental matrices, extraction of the biomarkers from the microbes, separation of the biomarkers from the other components, and detection of the biomarkers.

Each of these four components of the biomarker analysis will be examined in this review.

RESULTS AND DISCUSSION

Concentration of the microbes: The biomarker based method of BW agent detection when based on recovery of airborne microorganisms or their products from glass fiber filters has been shown to be much more effective than the classical standard culturing methods. Detection methods based on culturing the organisms retained on the membranes have been shown to be woefully ineffective in determining the actual number of microbes in indoor air samples [2,3]. The biomarker methods on the other hand where SLB analysis system is quantitative, allows detection of lipopolysaccharide immune potentiators, and through examination of the phospholipid fatty acid (PLFA) profile provides clear evidence of metabolic stress [2,3]. Through this research it became clear that the lipid extraction approach provides an excellent method for the detection and identification of airborne biohazards. The major limiting factors in this technique are the speed of extraction and detection.

Similar problems in the "infectious but not culturable" detection of pathogenic microorganisms have been encountered in the analysis of water and in the biofilms of drinking water distribution systems [3,4]. Again biomarker analysis has proven remarkable effective in the quantitative detection of unculturable pathogens and their immuomodulatring or allergenic products.

Extraction: The least developed and often the most time consuming step in the biomarker analysis utilizing lipids, nucleic acids or proteins is the extraction of the biomarkers. The initial step in lipid analysis classically involves a one-phase modified Bligh and Dyer solvent extraction for obtaining phospholipid fatty acid biomarkers used in community analysis. This method, however, is relatively labor intensive and slow, often taking up to 24 hours for the initial extraction.

Our laboratory has explored the utility of supercritical CO_2 (SFE-CO_2) extraction as a means of decreasing the time investment for SLB extraction procedures. SFE-CO_2 with methanol/ethanol enhancers has been shown to an excellent means to recover neutral lipids and pollutants from a wide variety of environmental matrices. We have shown that polar

phospholipids could not be extracted unless they were prederivatized with trimethylphenylammonium hydroxide (TMPH) to form the methyl esters *in situ* [6].

Nucleic acids, both DNA and rRNA are excellent biomarkers that provide specificity either as to the species or genera of the microbes or to the potential metabolic activity of specific gene products. The classical Bligh and Dyer room-temperature solvent extraction has been shown to be effective in facilitating the subsequent recovery of DNA and RNA from the same soil or membrane retentate samples from which the lipids have been extracted [7]. The DNA recovered in this system as been shown to be purified from some inhibitors of enzymatic amplification by PCR. Recently SFE-CO_2 with methanol/ethanol enhancers has been shown to be an excellent mechanism for cell lysis and recovery of DNA and RNA from a variety of environmental samples [8].

Lipids are probably the most readily extractable biomarkers. Accelerated solvent extraction (ASE) was used to extract SLB from selected vegetative and/or sporulated biomass as well as from environmental samples collected from water, soil, and air [9]. We have shown lipids extract efficiently ASE (at 1500 psi/80°C) with a 3-fold increase in recovery from bacterial spores, a 2-fold increase in recovery in fungal spores over room temperature/pressure solvent extraction [9]. ASE involved two 20-min cycles in contrast to the standard eight-hour room temperature extraction. Compared to the modified Bligh and Dyer extraction, phospholipid fatty acid (PLFA) lipid yields obtained using the pressurized hot solvent extraction were not significantly different for the vegetative biomass or water and soil samples, but were significantly higher for the spores and the air biomass samples. ASE can now be programmed to fractionate lipids. The system can be programmed to allow multiple extractions from the same sample so that an automated fractionation can be developed.

The increased extraction speed was achieved through a combination of increased pressure and temperature using the Dionex system. The Dionex system can process samples at a temperature of 200°C and pressures of 3,000 psi. with a minimum sample volume of 11 mL (using 18 mL minimum solvent). Experiments have shown that increasing the pressure and temperature of the extraction may considerably shorten the time for recovery of lipids from bacterial spores. Preliminary experiments showed lipid recovery from *Bacillus* spores in 5 min at 4500 psi/150°C in an ISCO SFE.

Proteins are probably the most difficult targets of extraction. Albumin has been extracted in supercritical CO_2 in a 1.4-% water emulsion with ammonium carboxylate perfluoropolyether surfactant [10], which changed the conformation of the protein but maintained its primary structure. These and other studies have established that it is possible to maintain a small water phase in supercritical CO_2 and that reactions such as reduction of manganese oxides and dyes can occur in the aqueous phase of emulsions in supercritical CO_2. We propose it should prove feasible to manipulate supercritical fluid extraction (SFE) and/or Accelerated Solvent Extraction (ASE) to rapidly isolate and fractionate lipids, DNA, and proteins for HPLC/ESI/MS/MS or IT(MS)^n.

Separation: The literature is rich in methods for the high performance liquid chromatographic (HPLC) separation and purification of lipids [11]. Moreover, polar lipids readily ionize under electrospray ionization conditions [12]. To facilitate the initial phases of

analysis, a C-18 coated open tubular capillary column between the extraction system and the electrospray ionization ion trap mass spectrometry system will provide in-line separation without the need for post-column splitting. This will provide for the class fractionation and quasi-molecular speciation of the polar lipid components. Electrospray ionization, widely regarded as the most versatile and sensitive method of ionization available, operating in the negative ion mode will provide information regarding the nature of the intact phospholipid, the identity of the two fatty acyl constituents, as well as that of the polar head group. As has been previously demonstrated, organic bases will be added at levels so as to enhance the electrospray signal of phospholipids components [13]. However, pH values greater than 8 have been demonstrated to rapidly deteriorate silica based columns.

Detection systems: The type of mass analyzer, which possesses both high sensitivity and size-weight-power-ruggedness reasonableness as a potential field detector is based on quadrupole, based systems. Quadrupole mass spectrometers can be either beam type, with a linear trajectory for the analyte ions, or ion trap quadrupoles, where the ions are stored in a "trap" and ejected for subsequent detection. The ion trap mass spectrometers (ITMS) have features particularly desirable for field instruments such as smaller size and lesser power requirements. The Teledyne ITMS has been chosen by the Army Environmental Center, Aberdeen, MD as its current field portable Tri-service Site Characterization and Analysis Penetrometer System (SCAPS) [14] and seems to be a reasonable first choice for the next generation CBW detector based on mass analysis.

Examples of threat organisms that have been detected by signature biomarker analysis: Lipid biomarker analysis has shown that many threat organisms have sufficiently unique signature lipid patterns making them detectable with potentially automatable rapid signature lipid biomarker analysis. Examples include *Bacillus* sp. spores [15], *Francisella tularensis* [16], 17 species of Mycobacteria [17], Legionella [18,19], and *Cryptosporidium parvum* [20,21].

Lipid biomarkers reflect an additional benefit besides the readily adaptation to rapid, automatable, and sensitive detection. Lipid patterns reflect the phenotypic responses of the microbes to the environmental conditions in which they have been growing [22]. This insight into the nutritional/physiological status can sometimes be reflected in the infectious potential or drug resistance of those pathogens. For example, in *Mycobacterium tuberculosis,* patterns of micoserosic acids and secondary alcohols derived from the surface waxes correlated with drug resistance when subjected to artificial neural network analysis[17]. With the protozoan pathogen *Cryptosporidium,* signature lipid biomarker analysis of environmental samples using high performance liquid chromatography/electrospray ionization/mass spectrometry (HPLC/ESI/MS) of lipid extracts allowed detection at a sensitivity of about 0.05 femtomoles of the unusual free fatty acid, 10-hydroxy stearate [22]. This is less than found in a single oocyst. The free fatty acid 10-hydroxy stearate is sufficiently unusual to be an excellent biomarker for this pathogen and has the enormous advantage of very possibly predicting infectiousness. Conditions which decrease the infectiousness of the oocysts to BALB/c neonatal mice such as incubation at 60°C for 8 hours, freezing at –70°C for 8 hours or exposure to pulsed ultraviolet light at 500 mWs/cm^2 decreased the 10-hydroxy stearate level 12-fold, 24-fold and > 1000-fold respectively[22]. Combining the rapid extraction, with HPLC/ESI/MS

signature biomarker analysis allows an automated analysis of this most difficult water-borne pathogen in from concentrated samples in less than an hour.

CONCLUSIONS

The combination of rapid and effective concentration technologies for aerosols and water with high pressure/high temperature extraction with HPLC/ESI/MS detection can provide a rapid, automatable, field-capable, BW detection system that is general and not specific to particular threat organisms and has the sensitivity to often not only provide detection but valuable insight into the infectiousness of the microorganisms. The specificity and application to toxins prions, and virus can be expanded by application to signature biomarkers if nucleic acids and proteins in addition to the lipids.

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